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High-throughput sequencing highlights the significant influence of seasonal housing and teat preparation on the raw milk microbiota

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Running title:

Factors influencing the raw milk microbiota

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Abstract

In pasture-based systems, changes in dairy herd habitat due to seasonality results in the exposure of animals to different environmental niches. These niches contain distinct microbial communities that may be transferred to raw milk, with potentially important food quality and safety implications for milk producers. It is postulated that the extent to which these microorganisms are transferred could be limited by the inclusion of a teat preparation step prior to milking. Here high-throughput sequencing, of a variety of microbial niches on the farm, is employed to study the patterns of microbial movement through the dairy production chain and, in the process, investigate the impact of seasonal housing and inclusion/exclusion of teat preparation regime on the raw milk microbiota from the same herd over two sampling periods, i.e., indoor and outdoor. Beta diversity and network analyses showed that environmental and milk microbiotas separated depending on whether they were sourced from an indoor or outdoor environment. Within these respective habitats, similarities between the milk microbiota and that of teat swab samples and, to a lesser extent, faecal samples were apparent. Indeed, SourceTracker identified the teat surface as the most significant source of contamination, with herd faeces being the next most prevalent source of contamination. In milk from cows grazing outdoors, teat prep significantly increased the numbers of total bacteria present. In summary, sequence-based microbiota analysis identified possible sources of raw milk contamination, and highlighted the influence of environment and farm management practices on the raw milk microbiota.

Importance

The composition of the raw milk microbiota is an important consideration from both a spoilage and food safety perspective and has implications for milk targeted for direct consumption and for downstream processing. Factors which influence contamination have been examined previously, primarily through the use of culture-based techniques. This manuscript describes the extensive application of high throughput DNA sequencing technologies to study the relationship between the milk production environment and the raw milk microbiota. Results highlight that the environment in which the herd was kept was the primary driver of the composition of the milk microbiota composition.

Introduction

The impact of the dairy farm environment on the microbial composition of raw milk and raw milk products has been appreciated for some time (1). There are numerous niches that collectively constitute the dairy farm environment and these harbour a vast array of microbes. The transfer of microbes from the farm environment to raw milk can be influenced be a number of factors including farmer hygiene, husbandry practices, herd health, and herd housing (2). In turn, the microbial composition of raw milk is critically important to its quality, processability and safety.

The microbiota composition of dairy farm niches and of raw milk has typically been examined using traditional plate cultivation-based techniques. These culture-based assays are still widely used by industry and target specific phenotypes, e.g. ability to grow at or survive exposure to particular temperatures (psychrotrophs (3), mesophiles (4), thermodurics (5), or capacity to produce proteases, lipases or other enzymes (6)) or species known to be human pathogens (2). Using these culture-based techniques, Vacheyrou previously examined possible routes of microbial transfer in farms supplying raw milk for Comte style cheese, revealing that the extent to which milk was contaminated varied depending on the type of barns used to house animals (2). However, recent advances in molecular microbiology, and in high throughput DNA sequencing (HTS) in particular, have allowed for a more in-depth analysis of the flow of microbes through environments (7-12).

Indeed, a study of two artisan cheese-making plants observed that spatial diversification within both plants was indicative of “functional adaptations” by microbial communities colonising different fomites within each plant. Spatial diversification between plants confirms the phenomenon of a unique production plant (“house”)-associated microbiota, which was postulated to influence the distinct organoleptic properties of products from each facility (11). The facility-specific microbiota developed as a result of the selection pressure introduced by the individual cheese-making processing methods (11). The observation of a niche-specific functional adaptation has also been observed in the microbiota of a winery, with the additional observation that the community was influenced by seasonality (12).

The present proof of concept study focuses on the Irish dairy farm system, which is primarily a pasture based system, in which herds are grazed on pasture for the majority of their lactation curve. However, during the winter months, herds are housed indoors. The transition between environments is an important consideration for dairy producers as it is accompanied by changes in exposure to microbes from different niches in the environment as well as dietary changes. Previous, culture-based, efforts to address this question have noted elevated spore counts in bulk tank milk collected from a number of mid-West American farms during summer months on American farms (13), although elevated numbers of sporeformers can also be an issue when cows are housed indoors if poor quality silage is used (14). Our study also investigates the impact that teat preparation has on the microbiology of raw milk. This farm management practice has been shown to reduce bacterial counts in milk previously (15) but its impact on the raw milk microbiota has not been reported.

Based on the results of the studies highlighted above, and in the context of the seasonal milk production system applied in Ireland (all cows calved within a 12 week period), it is reasonable to assume that cattle are exposed to niche-specific microbes when housed indoors during winter months, and that these environmental microbes differ significantly from that present when the herd is grazing on pasture during the summer. Such differences would be expected, in turn, to impact on the raw milk microbiota. Specifically, we examined the influence that seasonal housing and grazing conditions have on the microbiota of raw cows' milk. We also examined the influence that the farm management practice of teat preparation (prep) has on the raw milk microbiota in both environments. To address these questions, we applied HTS and a Bayesian inference algorithm to examine environmental sources of bacteria, as well as seasonal changes to the raw milk microbiota driven by changes in habitat.

Materials and methods

Treatment and Sample collection

106 Samples were collected from the same herd of Holstein-Friesian dairy cows (n=60) from the
107 Moorepark Research Farm (Fermoy, Co Cork, Ireland) during February (Average days in milk;
108 ADIM= 140) and May (ADIM=200)) of 2015. The milking parlour and equipment were
109 cleaned after each milking as outlined previously(16). Sampling phases corresponded to
110 when cows were housed indoors (February) and outdoors on pasture (May). During the
111 indoor sampling period (February) cows were fed grass silage within a cubicle house with
112 automatic scraper cleaning of the central passageway. Cubicle beds were fitted with rubber
113 mats with a daily allowance of ground limestone added to the backend of the cubicle. Cows
114 managed in the outdoor sampling period (May) grazed on perennial ryegrass pasture on a
115 24h rotational grazing regime. The herd was milked in a 30-unit, 80-degree side-by-side
116 milking parlour (Dairymaster, Causeway, Co Kerry, Ireland). Although most studies
117 incorporating molecular methods focus only on the bulk tank milk (BTM), in this instance,
118 milk from three individual cows was also tested. Three cows with a somatic cell count lower
119 than 100,000 cells/mL were chosen for specific individual sampling before commencement
120 of the study and were used throughout the study. Milk and teat swab samples were
121 collected twice weekly from these three cows throughout the study during the morning
122 milking.

123 Two pre-milking teat preparation treatments were applied within each sampling phase. One
124 treatment comprised of washing teats with running water, drawing of foremilk, and an
125 application of a pre-milking teat disinfectant (Deosan Teat-foam) (Deosan, Johnson Diversey
126 (Ireland) Ltd, Jamestown RD, Finglas 11, Dublin) followed at least 30 seconds later by drying
127 using individual paper towels, prior to attaching the milking cluster (prep). The second
128 treatment involved no teat preparation prior to cluster attachment for milking (non prep).
129 For both indoor and outdoor sampling periods, the teat treatments applied were as follows:
130 week one, all animals had teats prepped prior to milking; week two, animals were not
131 prepped; week three, teats were prepped prior to milking and week four no teat
132 preparation was carried out. All cows in the herd were subjected to each teat preparation
133 treatment at each day of sampling. Environmental samples (faeces, bedding, silage grass
134 and surface soil) were collected twice a week on day 1 and day 3, apart from the teat swab
135 samples, which were collected after the teat preparation treatment was applied and prior to
136 cluster attachment for milking on days 2 and 4. Microbial DNA was extracted from all

samples using the Powersoil kit (Mobio, Carlsbad CA). Due to the different sample types, the pre-processing protocol for samples varied. At morning's milking on day 2 and 4 of each sampling week, all four teats from the cows were swabbed using one sterile cotton swab per teat (Sarstedt, Ireland). Swabs were dipped in a solution of 3ml of NaCl (0.09%) prior to swabbing to improve recovery (17). Swabs were drawn across the teat orifice and up the side of each teat avoiding contact with the udder hair. The four swabs from each cow were then pooled in a NaCl solution (12 ml) in a sterile 15 ml falcon tube (Sarstedt, Ireland) and vortexed for 2 minutes. This resulted in one teat pool for each cow sampled at each time point. The pool, including liquid and swab heads, was then centrifuged for 5 minutes at 900 x g to separate the swab heads from the liquid. The supernatant was then removed and transferred to another sterile 15 ml falcon tube. Each pool was then centrifuged at 5444 x g for 30 minutes at 4 ^{o}C. The supernatant was then carefully removed and the resulting pellet was dissolved in the lysis solution from the Powersoil microbead tubes.

Milk samples from the selected three cows were collected within sterilized sampling bottles using the Weighall milk meter on days 2 and 4 of each sampling week (Dairymaster, Causeway, Co Kerry, Ireland). 60 ml of individual milk was used for each extraction. BTM samples representing the complete herd were collected after the morning milking on days 2 and 4. These were collected using 30 ml sterile blue dippa sample tubes (Ocon chemicals). 60 ml of the BTM was used for each extraction. For both individual milk and BTM, milk was aseptically transferred to 15ml Falcon tubes (Sarstedt, Ireland), and centrifuged at 5444 x g for 30 minutes at 4 ^{o}C. The fat layer was carefully removed and the supernatant was decanted. The resulting pellets were then washed using sterile PBS and centrifuged at 14,000 x g for 1 minute. The four pellets for each individual milk and BTM sample were then pooled, to give four samples (three individual milk samples and one BTM sample). Cell pellets were then dissolved in the lysis solution from the microbead tubes from the Powersoil kit.

For faecal pool samples, a pool of the herd's faecal samples was created at each day of sampling. Two faecal pools were collected on each week of sampling on day 1 and 3. To make this pool, equivalent amounts of faecal material were collected from 5 random cow pats and the pool was then homogenised for 2 minutes by vortexing at full speed. DNA was extracted from 250mg of this faecal pool.

Surface soil samples were collected on days 1 and 3 from the paddock from which the herd were grazing. These samples were collected, taking care to avoid collecting faeces or grass using a disposable spatula (VWR, Ireland),250mg of surface soil was used for the soil sample extractions. For bedding, silage and grass samples, 20 g of material was aseptically collected using sterile forceps (VWR, Ireland) and scissors (for grass samples) (Medguard, Co. Meath Ireland) and stored in stomacher bags. For bedding samples 4g of bedding material was collected from 5 cubicles from which the herd had been occupying to create a 20g bedding sample, two bedding samples were collected on each week of the indoor sampling period. For silage samples 20g of silage was collected from where the herd was feeding, two silage samples were collected on each week of the indoor sampling period. For grass samples, 20g of grass was aseptically collected from the paddock in which the herd had been grazing when outdoors; two grass samples were collected on each week of the outdoor sampling period. Then180 ml of sterile PBS was added to each stomacher bag and the samples were homogenised in a stomacher. The resultant mixture was then aliquoted into 50 ml falcon tubes and centrifuged at 900 x G for 5 minutes to remove solids. Following this, the supernatant was filtered through 0.45 uM nitro cellulose filter membrane (Merck Millipore). After filtration, the membrane was aseptically cut into microbead tubes (Powersoil kit) using a sterile scissors and forceps.

The sample numbers collected included surface soil (n = 8), faeces (n = 16, 8 indoor pools and 8 outdoor pools), silage (n = 8) and bedding (n = 8), as well as teat swabs (n = 48, of which 40 subsequently yielded amplicons - 10 indoor no prep [INP], 11 indoor prep [IP], 11 outdoor prep [OP] and 8 outdoor no prep [ONP]), individual milk samples (n=48, of which 47 subsequently yielded amplicons -12 INP, 12 IP, 11 OP and 12 ONP), bulk tank milk (BTM; n=14, 4 INP, 3 IP, 3 ONP, and 4 OP) and grass (n = 8).

After pre-processing of the samples had been pre-processed and lysis solution added, C1 solution lysis solution (preheated to 60°C) was added to all samples, and followed incubation for 10 minutes at 60°C with vortexing every two minutes for 30 seconds. After this incubation, samples were mechanically lysed at full speed for 10 minutes using a TissueLyser (Qiagen) and then processed as per Powersoil kit protocol. DNA was quantified and quality checked by gel electrophoresis and spectrophotometry on a nanodrop 1000 instrument (Thermo Fisher Scientific Inc).

16S rRNA amplicon sequencing

The V3-V4 variable region of the 16S rRNA gene was amplified from the 149 DNA extracts using the 16S metagenomic sequencing library protocol (Illumina). PCR reactions were completed on the template DNA. Initially, the DNA was amplified with primers specific to the V3-V4 region of the 16S rRNA gene which also incorporates the Illumina overhang adaptor (Forward primer 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG; reverse primer 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) (18). Each PCR reaction contained DNA template (~10–12ng), 5 µl forward primer (1 µM), 5 µl reverse primer (1 µM), 12.5 µl 2X Kapa HiFi Hotstart ready mix (Anachem, Dublin, Ireland), PCR grade water to a final volume of 25µl. For environmental samples (surface soil, faecal, silage, swabs, bedding, and grass) PCR amplification was carried out as follows: heated lid 110°C, 95°C x 3mins, 25 cycles of 95°C x 30s, 55°C x 30s, 72°C x 30s, then 72°C x 5mins and held at 4°C was used. For milk samples the same cycling parameters were used, accept 32 cycles were used instead of 25 cycles. PCR products were visualised using gel electrophoresis (1X TAE buffer, 1.5% agarose, 100V) and cleaned using AMPure XP magnetic beads (Labplan, Dublin, Ireland). Following this, a subsequent PCR reaction was completed on the purified DNA (5µl) to index each of the samples, allowing samples to be pooled for sequencing on three flow cell and subsequently demultiplexed for analysis. Samples were indexed randomly to prevent any run bias in analysis. Two indexing primers (Illumina Nextera XT indexing primers, Illumina, Sweden) were used per sample. Each PCR reaction contained 5µl index 1 primer (N7xx), 5µl index 2 primer (S5xx), 25µl 2x Kapa HiFi Hot Start Ready mix, 10µl PCR grade water. PCRs were completed as described above, with 8 amplification cycles. PCR products were visualised using gel electrophoresis and subsequently cleaned (as described above). Samples were quantified using the Qubit (Bio-Sciences, Dublin, Ireland); along with the broad range DNA quantification assay kit (BioSciences) and samples were then pooled in an equimolar fashion. The pooled sample was run on the Agilent Bioanalyser for quality analysis prior to sequencing. The sample pool (4nM) was denatured with 0.2N NaOH, then diluted to 4pM and combined with 10% (v/v) denatured 4pM PhiX, prepared following Illumina guidelines. Samples were sequenced on the MiSeq sequencing platform in the Teagasc sequencing facility, using a 2 x 250 cycle V3 kit, following standard Illumina

sequencing protocols. Reads were deposited in the SRA database under the accession number PRJEB16770.

Bioinformatic and statistical analysis

250 base pair paired-end reads were assembled using FLASH (FLASH: fast length adjustment of short reads to improve genome assemblies) (19). Further processing of paired-end reads including quality filtering based on a quality score of > 25 and removal of mismatched barcodes and sequences below length thresholds was completed using QIIME(20). A total of 32,766,563 reads were generated post filtering, with an average of 219,909 per sample. Denoising, chimera detection and clustering into operational taxonomic units (OTUs) (97% identity) were performed using USEARCH v7 (64-bit)(21). OTUs were aligned using PyNAST (python nearest alignment space termination; a flexible tool for aligning sequences to a template alignment) and taxonomy was assigned using BLAST against the SILVA SSURef database release 111. Samples were then rarefied to an even depth of sequences per sample. Alpha diversity was generated in QIIME and the compareGroups function (22) was then was then used to determine any statistically significant differences (P=<0.05) and generate standard deviations between samples based on conditions using the ANOVA test.. Beta diversity was calculated in R, using Phyloseq (23) and Bray Curtis distances.Principal coordinate analysis (PCoA) plots were visualised using ggplot2 (24). Confidence ellipses were generated using stat_ellipse in the ggplot2 package (24). Network analysis was also carried out using phyloseq and ggplot2. The SourceTracker algorithm (9) was also used to investigate possible sources of environmental contamination in milk from both sampling periods. SourceTracker analysis was carried out at a depth of 13500, with 100 burn-ins and 10 re-starts. The compareGroups function was used in R to compare differences in microbial composition between individual milk, teat swab and faecal pool samples; the Kruskal Wallis test was applied in this instance with Benjamini-Hochberg corrections (25), to highlight any statistically significant differences (P=<0.05 after correction).

Quantitative PCR

Quantitative PCR (qPCR) was carried out on individual milk samples to determine total bacteria levels in each sample using 16S rRNA gene. qPCR was carried out as described previously (26) except for the use of the equivalent volume of Kappa SYBR fast (Roche

Diagnostics) was used instead of SYBR green for the present study. Samples, negative controls (where template DNA was replaced with PCR-grade water) and standards were run in triplicate (technical replicates).

Results

Microbiota alpha and beta diversity of raw milk, teat surface swabs and environmental samples cluster according to habitat

Samples were collected from the same herd over two sampling periods. Sampling phases corresponded to when the herd was housed indoors and outdoors on pasture, respectively. Across both sampling phases, milk samples were collected from teat prepared (prepped) and non-teat prepped samples. Samples were also classified as either a potential 'source' of microorganisms or a 'sink' (a sample that is liable to contain bacteria originating from a source). Milk samples both from individual cows and BTM were classified as sinks and all environmental samples were classified as sources. After sequencing, the alpha and beta diversity of the bacterial populations present was investigated.

Alpha diversity is the diversity in each sample, using species richness and evenness to calculate the diversity in each environment. There was no significant difference in alpha diversity between the microbiotas of individual indoor and outdoor milk samples from non-prepped animals. Similarly, there was no significant difference in the alpha diversity of the microbiota of indoor milk sourced from animals who underwent teat prep and those that did not. However, the alpha diversity of the outdoor milk microbiota was significantly higher in OP samples relative ONP ($P=0.016$ Simpsons diversity index, $P=0.008$ Shannon diversity index; Table 1). A corresponding analysis of the alpha diversity of the microbiota of the teat surface revealed significantly greater diversity (chao1, Shannon, PD whole tree and observed species) among OP samples relative to IP samples ($P=<0.01$, 0.026, <0.01 and <0.01, respectively; Table 1). No other significant differences in the alpha diversity of teat microbiota samples were observed.

Beta diversity is the diversity between different samples; it provides a measure of dissimilarity between samples. The Bray Curtis Principle Coordinate plot of beta diversity

(Fig.1A) depicts all samples from this study with data points coloured by sample origin and shaped according to their designation as source or sink. In this plot it can be observed that samples (soil, grass, bedding, silage, teat surface indoor, teat surface outdoor, faecal indoor pool, faecal outdoor pool, indoor milk, outdoor milk [individual and BTM]) form clusters, which in turn are further separated from one another based on habitat (outdoor/indoor). More specifically, there is a clear separation between samples depending on whether they were collected from an indoor or an outdoor environment. Faeces, teat, individual milk samples and BTM samples also separate based on which environment they were sampled from (indoor/outdoor) (Fig.1A). There are more similarities between samples taken from the same habitat. This includes environmental samples (grass and soil [outdoor] and bedding and silage [indoor]), as seen by the overlaps in the ellipses. Within both habitats, it is apparent that there is an overlap between data points representing the milk sample microbiota and that of teat swab samples, reflecting similarities in their beta diversity (Fig.1A). Teat prep did not result in further sub-clusters within the milk or teat samples (Fig.S1). Faecal pool samples from both habitats separate from one another and are located in relatively close proximity to the corresponding milk and teat samples from the same environment (Fig.1A).

Network analysis shows relationships between raw milk and environmental samples

Network plots are a useful graphical tool to illustrate relationships between microbiota datasets. The nodes in this network plot represent samples, and the edges that connect nodes indicate correlations between samples. The network analysis shows relationships that exist between the environmental samples and milk samples (Fig.1B). Consistent with beta diversity data, it is particularly notable that, of the environmental microbiota samples, the faecal pools and teat microbiota are most closely related to the microbiota of the milk samples, thereby identifying faeces and the teat surface as important sources of contamination. These relationships reflect the habitat (indoor or outdoor) from which the samples were collected. There are more edges linking indoor faecal pool samples with indoor BTM samples, than outdoor faecal pool samples with outdoor BTM. Some of the outdoor milk samples are not linked to any of the outdoor sources by edges. This suggests that these niches are not substantial sources of microbial contaminants in these milk samples.

SourceTracker analysis further highlights the contribution of faecal and teat sources to the raw milk microbiota

The SourceTracker model assumes that each individual community (milk, soil, grass, faeces, teat, bedding and silage) is a mixture of communities deposited from other known or unknown source environments and, using a Bayesian approach, the model provides an estimate of the proportion of the community originating from each of the different sources. When a community contains a mixture of taxa that do not match any of the potential source environments studied, that portion of the community is assigned to an "unknown" source. The analysis revealed that the teat surface was the most significant contributor of microbes in milk samples regardless of habitat or teat preparation. Teat surface contaminants constitute a higher proportion of total contaminants in indoor milk compared to outdoor milk, both for individual and for BTM samples. Faeces was the next most important source of contaminants, and had a greater influence on indoor, than outdoor, milk samples, particularly in BTM samples (Fig.2).

Taxonomic analysis of raw milk, teat surface and herd faecal microbiota

Graphs representing the microbiota at Family level in the various sample sets are provided in the supplementary data (Supplementary Fig. 2-3). The compareGroups function was used in R to compare differences in microbial composition between samples. OTUs that differ significantly can be found in the supplementary material (Tables S1-S3). In milk samples from individual animals that did not undergo a teat prep treatment, it was noted that indoor samples contained higher relative proportions of, for example, *Eremococcus*, *Ruminococcus*, *Prevotella*, uncultured *Corynebacteriales* bacterium, and *Ruminococcaceae* Incertae Sedis (P=0.012, 0.012, 0.02, 0.022, 0.028, respectively) and lower proportions of *Pseudomonas*, *Acinetobacter, Lactococcus* and *Tumebacillus* (P=0.003, 0.008, 0.002 and 0.014 respectively), relative to outdoor milk samples. qPCR analysis to determine total bacterial numbers showed that there was significantly more bacteria in indoor milk samples than the equivalent outdoor milk samples (P=0.003) (Table 2). When the corresponding milk samples from individual teat prepped animals were compared, it was noted that 25 genera were present in significantly different proportions in indoor milk samples relative to outdoor-milk samples. Sixteen of these OTUs were higher in indoor samples, these include *Eremococcus*,

350 *Alloiococcus*, *Trichococcus*, *Prevotella*, and *Psychrobacter*, which were all more abundant in
351 indoor samples (P=0.001, 0.001, 0.001, 0.02, and 0.019, respectively). Nine OTUs were
352 higher in PO samples, including *Flavobacterium*, *Sphingomonas* and *Tumebacillus* (P= 0.009,
353 0.014, and 0.021 respectively). There was no significant difference in total bacterial numbers
354 between the indoor and outdoor milk samples from teat prepped cows (P=0.598) (Table 2).

355 The taxonomic data also facilitated an analysis of the specific effects of teat prep on the
356 bacterial composition of the milk produced. In indoor milk samples from individual animals,
357 it was noted that proportions of *Pseudomonas* were higher in samples from cows which had
358 undergone teat prep (P=0.035) suggesting that, among the indoor teat microbiota,
359 *Pseudomonas* was relatively less sensitive to the antimicrobial effects of the teat prep in
360 indoor samples. qPCR analysis demonstrated that there was no significant difference in total
361 bacterial numbers because of the teat prep (P=0.758) (Table 2). *Pseudomonas*, *Lactococcus*
362 and *Lactobacillus* were among nine genera present in outdoor milk samples that were
363 influenced by teat prep. In the case of the aforementioned genera, proportions were higher
364 in samples when no teat prep was carried out (P=0.011, 0.025, and 0.03, respectively).
365 There were significantly fewer total bacteria in milk samples from non-prepped animals
366 samples compared to samples from prepped animals in the outdoor environment (P=0.004)
367 (Table 2).

368 The microbiota composition of the teat swabs was also assessed and it was established that,
369 in samples where teat prep did not occur, 18 genera differed significantly in their relative
370 abundance between indoor and outdoor samples. *Trichococcus, Proteiniphilum, and*
371 *Eremococcus*, as well as *Corynebacterium*, were more abundant in indoor samples (P= 0.012,
372 0.021, 0.044, and 0.039, respectively) while a further 11 OTU's were present in significantly
373 higher proportions in outdoor samples. In samples where teat preparation was carried out,
374 60 genera differed significantly between indoor and outdoor samples. Twenty-one of these,
375 including *Eremococcus*, *Proteiniphilum*, *Corynebacterium*, *Psychrobacter Bifidobacterium*,
376 *Trichococcus* and *Prevotella*, were significantly higher in indoor samples (P= 0.001, 0.001
377 0.002, 0.002 0.003, 0.004, and 0.005, respectively) and thirty-nine genera, including
378 *Stenotrophomonas*, *Xanthomonas* and *Rhizobium*, (P= 0.001, 0.001, and 0.003, respectively)
379 were significantly higher in outdoor samples. Among the outdoor teat samples, there were
380 no significant differences between prepped and non-prepped samples. Among the

corresponding indoor teat samples, proportions of *Variovorax* and *Devosia* were higher in teat samples which were not treated (P=0.033 and 0.043) (Supplementary table 2).

Additionally, it is noteworthy from the stacked bar charts (Fig S1 (B) and (D)) that the composition of individual milk samples differs considerably from that of BTM. More specifically, higher proportions of *Micrococcaceae* and *Flavobacteriaceae* are observed in all individual milk sample types and *Prevotella* and *Rikenellaceae* were higher in BTM samples.

Finally, the availability of faecal pool samples from both the indoor and outdoor environment facilitated a comparison of their composition. At the genus level 15 genera, including *Prevotella*, *Bacteroides* and *Treponema*, were higher in indoor faecal pool samples (P=0.001, 0.002, and 0.021) and a further eight genera, including *Phocaeicola* and *Paludibacter*, were higher in outdoor faecal pool samples (P=0.027 and 0.036) (Supplementary table 3).

Discussion

The objective of this proof of concept study was to harness the power of next-generation DNA sequencing technologies to investigate the influence that seasonal housing and teat preparation have on the raw milk microbiota from individual cows and in BTM. Furthermore, information potentially revealing the extent to which different microbial niches in the milk production environment influence the microbiota of raw milk was also generated. While, in the past, culture-based investigations to study the source of microorganisms in raw milk have primarily focused on BTM, in this instance samples from a small subset of individual animals was also included. While analysis did not reveal differences between the microbiota alpha diversity of indoor and outdoor milk samples, beta diversity analysis highlighted a clear separation between samples that are sourced from an indoor versus an outdoor environment. No distinct separation pattern was observed when samples were coloured by teat preparation treatment (Fig S1). Thus, this analysis demonstrates that habitat had a greater impact on the raw milk microbiota than teat preparation.

409 The SourceTracker algorithm was used as a complementary means of identifying the likely
410 source within the dairy farm environment (soil, silage, bedding, grass, teat, and faeces) of
411 bacteria ultimately found in raw milk and, in the process, also reveals the influence of
412 seasonal housing and farm management practices. Regardless of habitat or treatment, teat
413 surface was again identified as the greatest contributor to the raw milk microbiota, followed
414 by faeces. This is consistent with a previous (culture-based) study, which proposed that the
415 teat skin was a source of microbial populations in raw milk and that farm management and
416 animal grazing practices influenced the diversity and microbiota of raw milk(27).

417 The taxonomic results also show that habitat had a much greater influence on the raw milk
418 and teat microbiota than teat prep. For instance, in milk samples from cows that were not
419 subjected to teat prep, Gram positive and gut-associated genera were higher in indoor,
420 relative to outdoor milk, such as *Ruminococcus*, *Eremococcus*, *Ruminococcaceae* Incertae
421 Sedis and uncultured *Corynebacteriales* were higher in indoor, relative to outdoor, samples.
422 *Ruminococcus* and *Ruminococcaceae* Incertae Sedis are both gut-associated genera
423 although, from a dairy perspective, *Ruminococcaceae* Incertae Sedis has previously been
424 found in continental type cheese (28) and *Ruminococcus* has been detected in raw milk (29)
425 , and in this study these were in higher proportions in INP milk compared to ONP. While,
426 relatively little is known about the uncultured *Corynebacteriales*, the cultured equivalent
427 contains species known to cause mastitis (30) as well as others that are found on the surface
428 of surface-ripened cheese (31). Similarly, the other genus noted, *Eremococcus*, has not been
429 well characterised, although a typed strain does exist, having been isolated from the vaginal
430 discharge of a thoroughbred horse (32). Proportions of the Gram negative genus *Prevotella*,
431 which is typically gut-associated was also higher in indoor samples while, for the outdoor
432 samples, the Gram negative genera *Pseudomonas* and *Acinetobacter*, as well as the Gram
433 positive genus *Lactococcus*, were among those that were more dominant. *Pseudomonas*
434 and *Acinetobacter* are both dairy spoilage-associated genera (6) that can have a negative
435 impact on dairy product quality. Lactococci are best known for their positive contribution to
436 the production of fermented dairy products, but can also be isolated from outdoor
437 environments such as grass (33). These results indicate that indoor milk is more likely to
438 have higher proportions of host/gut associated microbes than outdoor milk while,

439 unsurprisingly, outdoor milk is more likely to contain higher proportions of environmental
440 bacteria.

441 For milk samples from cows that were teat prepped prior to milking, LAB, such as
442 *Eremococcus*, *Alloiococcus*, and *Trichococcus,* as well as *Psychrobacter*, are also in a
443 significantly higher proportion in IP samples. Interestingly, *Alloiococcus* has not been
444 described in raw milk previously, having instead being associated with human ear infections
445 (34). *Trichococcus* has been found in raw milk and dairy waste (35) and *Psychrobacter* have
446 previously been found in teat apexes (36) and in cheese (37). Again, in the corresponding OP
447 milk samples soil bacteria such as *Flavobacterium*, *Sphingomonas* and *Tumebacillus* where
448 in higher proportions. This indicates that outdoor milk is more likely to contain increased
449 proportions of soil associated microbes, while indoor milk is more likely to have higher
450 proportions of host/gut bacteria. The proportions of LAB found in the milk appear to be low
451 in comparison to other studies (29) , this is perhaps due to the protocol used which did not
452 incorporate enzymatic lysis.

453 In teat swab samples, Gram positive genera such as *Corynebacterium*, *Trichococcus* and
454 *Eremococcus* and Gram negative genera such as *Proteiniphilum* were significantly higher in
455 NPI samples compared to NPO samples. *Proteiniphilum* has previously been associated with
456 the faeces of dairy cattle (38). A number of soil type OTU's were observed to be significantly
457 elevated in NPO, relative to NPI teat swab samples. This indicates that the transmission of
458 soil type bacteria to the teat is greater in periods where cows are grazing outdoors,
459 potentially leading to subsequent transmission from the teat to milk. In teat samples that
460 were prepped, *Corynebacterium*, *Eremococcus* and *Trichococcus* were again more abundant
461 in IP teat samples. *Bifidobacterium* was also present in greater proportions in these samples.
462 Although *Bifidobacterium* is typically associated with the gastrointestinal tract (GIT) of warm
463 blooded mammals (38), it may be significant that prep has previously been shown to cause
464 an increase in Actinobacteria proportions on the teat surface (15). With regard to Gram
465 negative bacteria, *Proteiniphilum*, *Psychrobacter* and *Prevotella*, were all significantly more
466 abundant in IP teat swab samples compared to OP samples. In outdoor samples that were
467 teat prepped, many soil type bacteria, including *Rhizobium*, *Xanthomonas*, and
468 *Stenotrophomonas*, were significantly more prevalent compared to OP samples. Thus, soil-

type bacteria, also noted on the surface of ONP teat surface, persist even when teat prep occurs.

Using the data generated, it possible to assess the impact of teat preparation on the milk and teat microbiota composition by comparing data from animals that were/were not subjected to a treatment (during the same season). In milk samples, lactic acid bacteria, such as *Lactococcus* and *Lactobacillus,* and *Pseudomonas* were higher in NPO samples, suggesting that the application teat prep significantly reduced the numbers of these microbes in raw milk. There were no significant differences between PO and NPO teat swab samples. Among indoor teat samples, soil type *Proteobacteria*, such as *Variovorax* and *Devosia*, were more abundant in NPI, relative to PI teats. *Variovorax* has previously been found in hay (2), and *Devosia* has previously been found in raw milk (39). It was surprising to note that teat prep increased the numbers of total bacteria in both indoor and outdoor milk. Alpha diversity was also found to have increased in milk from cows where teats were prepped prior to milking compared to milk from cows where teat preparation was omitted. It may be that the teat preparation process, including forestripping and drying, weakens the attachment of commensal and contaminating teat canal bacteria and results in their being shed into the milk in greater numbers. This result contrasts findings from culture based analysis on the impact of teat prep on raw milk, which found that it reduced bacterial diversity or counts respectivly (15, 40). Further studies will be required to re-examine the influence that teat preparation has on the raw milk microbiota. Another important consideration is that the farm used in this study is a research farm where stringent hygiene practices are upheld. This could perhaps limit the impact that teat preparation has on the raw microbiota

There were considerable differences observed between the individual milk and BTM microbiotas (Fig S1). This may be due to microorganisms in the BTM being acquired from the milking machine and pipes. Indeed, this possibility has been highlighted previously (41) but not in the context of DNA-based analysis. Further explorations to definitively establish the basis for these differences is merited.

The availability of faecal microbiota data from multiple samples also facilitated comparative analysis of these samples. It was apparent that the beta diversity of the herd faecal pool

499 microbiota differed significantly from the two sampling periods. From a taxonomic
500 perspective, eight genera were found to be significantly higher in outdoor herd faecal
501 samples and fifteen genera were found to be significantly higher in indoor herd faecal pool
502 samples. *Treponema*, *Prevotella* and *Bacteroides* were among the gut-associated genera
503 that were more prevalent in indoor samples. *Treponema* has previously been associated
504 with digital dermatitis in cattle (42) and in the bovine rumen (43). *Phocaeicola* and
505 *Paludibacter* have also been positively associated with valerate in the rumen previously (44),
506 and were higher in outdoor samples. This difference in faecal microbiota may be influenced
507 by habitat, host physiological changes or by dietary changes associated with the differing
508 habitats. It is also possible that transmission of bacteria from faecal origin may differ based
509 on habitat due to the differences in the microbiota seen here.

510 Here, high-throughput DNA sequencing has facilitated the analysis of the microbiota of raw
511 milk samples in parallel with samples from the dairy farm environment. The results provide
512 a more detailed insight into the composition of these microbial populations while also
513 allowing an examination of the relationship between the microbiota of these environments
514 and of raw milk. This analysis highlights that herd habitat is a significant driver for milk
515 microbiota composition, and that teat prep has a much more limited impact on the raw milk
516 microbiota. In the process it is made apparent that high-throughput sequencing can be an
517 extremely insightful tool to help better understand the movement of microbes from the
518 environment into the food chain.

519

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650 its relationship with the concentration of volatile fatty acids in the feces during subacute
651 rumen acidosis in dairy cows. BMC Veterinary Research **8:**1-13.

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654 **Table 1: Alpha diversity differences between individual milk and teat swab samples**

Milk								
	INP	**ONP**	**IP**	**OP**	**P value INP vs ONP**	**P value IP vs OP**	**P value INP vs IP**	**P value ONP vs OP**
chao1	3139 (1271)	2733 (833)	3017 (703)	3328 (784)	0.721	0.867	0.99	0.445
Simpson	0.98 (0.02)	0.95 (0.05)	0.98 (0.02)	0.98 (0.02)	0.036	0.885	0.983	0.016
Shannon	8.25 (1.07)	7.49 (1.17)	8.26 (1.07)	9.02 (0.80)	0.309	0.361	1	0.008
PD whole tree	90.3 (29.4)	70.5 (27.2)	93.8 (26.1)	86.3 (23.8)	0.304	0.918	0.99	0.521
observed species	2914 (1232)	2525 (784)	2791 (706)	3036 (752)	0.726	0.922	0.988	0.547
Teat								
	INP	**ONP**	**IP**	**OP**	**P value INP vs ONP**	**P value IP vs OP**	**P value INP vs IP**	**P value ONP vs OP**
chao1	3373 (792)	4307 (1172)	2949 (536)	4791 (1219)	0.187	<0.001	0.742	0.699
Simpson	0.99 (0.01)	0.99 (0.00)	0.99 (0.01)	0.99 (0.00)	0.99	0.716	0.962	0.997
Shannon	8.54 (0.67)	8.84 (0.41)	8.44 (0.48)	9.17 (0.67)	0.695	0.026	0.977	0.612
PD whole tree	125 (27.0)	157 (37.7)	107 (17.8)	174 (39.9)	0.156	<0.001	0.589	0.665
observed species	3194 (767)	4090 (1119)	2725 (500)	4526 (1188)	0.19	<0.001	0.655	0.741

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656 Numbers in the brackets represent standard deviations. NPI= No prep indoor; NPO=No prep
657 outdoor; PI= Prep indoor; PO= Prep outdoor

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Table 2: (A)qPCR determination of total bacteria numbers for individual milk samples, (B) results of comparison total bacterial numbers present in individual milk samples from different conditions

A	Sample Type	Total bacteria (copies of 16S rRNA gene)
	INP	335500
	IP	424333
	ONP	49600
	OP	416000

B	Comparison	P values
	INP vs IP	0.758
	INP vs ONP	0.003
	IP vs OP	0.598
	ONP vs OP	0.004

NPI= No prep indoor; NPO=No prep outdoor, PI= Prep indoor, PO= Prep outdoor

679 Figure1 : (A)Bray-Curtis PCoA plot of milk and environmental samples, (B) Bray-Curtis Network plot
680 of milk and environmental samples. SourceSink indicates if a sample is classified as a potential
681 source of contamination or a sink for contaminating communities. ENV_dif indicated the sample
682 origin

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707 Figure2: SourceTracker results highlight the percentages of inferred sources of contamination in
708 BTM and individual milk samples

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